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Recognition of Psychostimulants, Antidepressants, and Other Inhibitors of Synaptic Neurotransmitter Uptake by the Plasma Membrane Monoamine Transporters

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ABSTRACT

The plasma membrane monoamine transporters terminate neurotransmission by removing dopamine, norepinephrine, or serotonin from the synaptic cleft between neurons. Specific inhibitors for these transporters, including the abused psychostimulants cocaine and amphetamine and the tricyclic and SSRI classes of antidepressants, exert their physiological effects by interfering with synaptic uptake and thus prolonging the actions of the monoamine. Pharmacological, biochemical, and immunological characterization of the many site-directed, chimeric, and deletion mutants generated for the plasma membrane monoamine transporters have revealed much about the commonalities and dissimilarities between transporter substrate, ion, and inhibitor binding sites. Mutations that alter the binding affinity or substrate uptake inhibition potency of inhibitors by at least 3-fold are the focus of this review. These findings are clarifying the picture regarding substrate uptake inhibitor/transporter protein interactions at the level of the drug pharmacophore and the amino acid residue, information necessary for rational design of novel medications for substance abuse and a variety of psychiatric disorders.

KEYWORDS: transporter, neurotransmitter, antidepressant, addiction, cocaine

INTRODUCTION

Plasma membrane transporters constitute the primary mechanism for synaptic clearance of neurotransmitter following Ca^{2+}-mediated exocytosis from synaptic vesicles. These proteins are responsible for translocating the cognate neurotransmitter from the extracellular space into the cytoplasm, at which point the neurotransmitter may be packaged into synaptic vesicles and recycled. The dopamine transporter (DAT), norepinephrine transporter (NET), and serotonin transporter (SERT) comprise the plasma membrane monoamine transporters, a subfamily that has been associated with psychostimulant actions and abuse, Parkinson's disease, attention deficit hyperactivity disorder, schizophrenia, narcolepsy, Lesch-Nyhan disease, postural hypotension, anxiety-related disorders, autism, and depression.[1-14] The plasma membrane monoamine transporters have been and will continue to be important therapeutic targets. Structure-function studies on the DAT, NET, and SERT can only increase the precision of rational drug design in treating the conditions mentioned above.

The plasma membrane monoamine transporters are members of the 12 transmembrane (TM) domain neurotransmitter:sodium symporter (NSS) family,[15] in which electrogenic transport of a neurotransmitter substrate across the cell membrane is driven by the naturally occurring neuronal Na^+ gradient. Cotransport of Cl^- is also required for the DAT, NET and SERT; the SERT additionally transports K^+, but in antiport fashion.[16] Aligning the amino acid sequences of the NSS family members guides delineation of monoamine transporter TM domain borders and other aspects of transporter secondary structure (Figure 1, Table 1).[17] Such a sequence alignment can also yield clues as to which monoamine transporter amino acid residues probably contribute to the general protein infrastructure, which residues may play a role in substrate or ion recognition, and which residues are most likely to be responsible for a pharmacologic pattern unique to a given transporter. One problem in comparing pharmacologic findings between site-directed mutants of different transporters is that each residue of a given transporter is named according to its position in the polypeptide chain. Thus, only from the sequence alignment is the TM 1 aspartic acid residue D79 in the human DAT revealed to be at the position analogous to D75 in the human NET and D98 in the human SERT. Fortunately, a new nomenclature for identifying residues with respect to their relative position in a given NSS TM domain has been developed recently[17] and is employed here. The most conserved residue in a given TM domain is arbitrarily assigned Position 50; this number is preceded by the TM domain number. As an example, because W84 of hDAT TM 1 is the most conserved residue in this TM domain among the NSS

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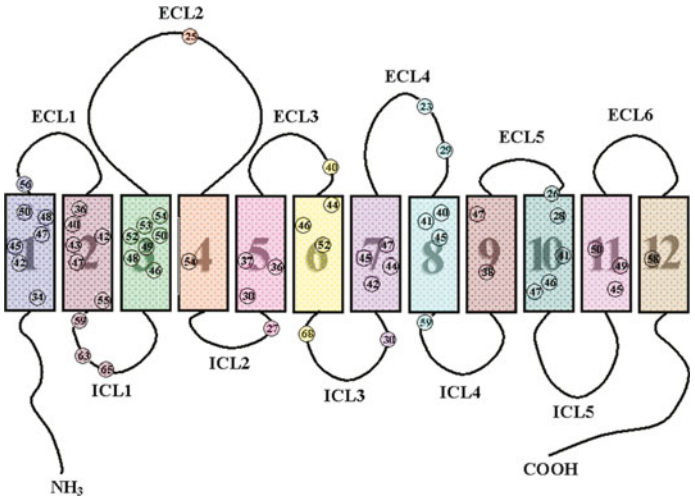

Figure 1. Helical net topology scheme for discussed monoamine transporter mutations. The 12 TM domains are indicated by cylinders, color-coded to match the TM domain assignments of specific mutations listed in Table 1. Numbers inscribed in circles indicate specific positions of TM residues using the indexing system of Goldberg et al[17]; amino acid side chain assignments are listed in Table 1. Thus, the circled "34" in TM 1 refers to Position 1.34 in Table 1, in turn indicating that aspartic acid residues are found at this analogous position for the human DAT, NET, and SERT. Mutagenesis targets in the 6 extracellular loops (ECLs) and 5 intracellular loops (ICLs) are color-coded to indicate which of the flanking TM domains is used in the indexing system. Thus, the hDAT E218 residue in ECL 2 has been assigned Position 25 of TM 4 and is referred to as $E218_{4.25}$ in the text.

proteins, this residue is termed $W_{1.50}$. V83, the hDAT residue immediately N-terminal to W84, is thus labeled $V_{1.49}$, and the immediately C-terminal R85 residue is labeled $R_{1.51}$. To facilitate the transition between the conventional and new nomenclatures, both numbering systems will be used (eg, $W84_{1.50}$), consistent with the suggestion of Weinstein, Javitch, and colleagues.[17]

Hundreds of plasma membrane monoamine transporter mutants have been constructed and characterized, but the large majority are not discussed here. This review focuses on discrete transporter residues that may contribute to recognition of inhibitors of substrate uptake. In this context, mutations of putative substrate binding site residues may also be discussed. NSS mutants for which the binding affinity or substrate uptake inhibition potency of uptake blockers is not altered by at least 3-fold are typically not discussed. Mutations that are believed to exert their effects via conformational changes or by altering the equilibrium between conformations are not emphasized but are discussed when the ≥ 3-fold pharmacologic shift was present. Mutants that displayed pronounced functional deficits and for which the extent of cell surface expression was ambiguous are not included. Regarding trafficking of mutants to the cell surface, wildtype-like B_{max} values for a ligand not expected to penetrate the plasma membrane (eg, the WIN 35,428 cocaine analog) were taken as proof of adequate cell surface expression.[18] Finally, findings from chimeric transporters are generally covered only when the study led to identification of specific residues critical for recognition of uptake inhibitors. To facilitate comparisons of specific mutations between monoamine transporters, the pharmacology of mutants in analogous positions are discussed one TM domain at a time.

Review

Transmembrane Domains

TM 1

A TM 1 aspartic acid residue common to the plasma membrane monoamine transporters but not shared by other NSS family members is the side chain perhaps most frequently postulated to directly contact cocaine and other nonsubstrate inhibitors, as well as substrates. This $D_{1.45}$ residue was the first to be mutated among NSS transporters,[19] an approach based on the G protein-coupled receptor model of a salt bridge between the positively charged agonist amino group and negatively charged TM domain carboxylate side chain.[20]

Consistent with the "salt bridge" premise, alanine, glycine, or glutamic acid substitution of rDAT $D79_{1.45}$ was reported to markedly decrease DAT affinities for dopamine and the cocaine analog WIN 35,428.[19] (The letters "h," "r," "m," "b," or "d" preceding the transporter name refer to the human, rat, mouse, bovine, or *Drosophila* species of that transporter, respectively.) On the other hand, recent findings with rDAT $D79_{1.45}E$ indicated no effect on dopamine affinity, only 3-fold losses in WIN 35,428, mazindol and methylphenidate affinities, and no effect on the dopamine uptake inhibition potency (DUIP) for these drugs.[21] Binding affinities and DUIPs for benztropine and its analogs were nevertheless typically altered substantially by this mutation.[22] The authors of the latter 2 studies question whether $D_{1.45}$ of the plasma membrane monoamine transporters is a logical counterion for the positively charged substrate amino group. A glycine side chain ($G_{1.45}$) is found in NSS family members including transporters for GABA, betaine, glycine, and proline, members whose cognate substrates share with the monoamines the positively charged amino group but lack aromatic groups.[21] The coincidence of an aspartic acid side chain at Position 1.45 in only those transporters recognizing aromatic substrates may indicate that $D_{1.45}$ serves as a strut supporting an aromatic binding site for the ligand.[22] Findings were inconsistent with, but did not rule out, formation of a salt bridge between $D_{1.45}$ and either dopamine, cocaine, or amphetamine.[21,22] $D75_{1.45}$ of the NET was intolerant to mutation, and like the DAT, only glutamate substitution of

Table 1. Discussed Amino Acid Residue Mutagenesis Targets and Their Counterparts at Analogous Positions in the DAT, NET, and SERT*

	1.34	1.42	1.45	1.47	1.48	1.50	1.56	2.36	2.40	2.42	2.43	2.47	2.55	2.59
DAT	D68 (h)	F76 (r)	D79 (r)	A81 (h)	N82 (h)	W84 (h)	C90 (h)	F98 (r)	Y102 (h)	L104 (m)	F105 (m)	A109 (m)	E117 (h)	G121 (h)
SERT	D87 (h)	Y95 (h)	D98 (h)	G100 (h)	N101 (h)	W103 (h)	C109 (h)	F117 (h)	Y121 (h)	I123 (h)	M124 (r)	G128 (h)	E136 (h)	G140 (h)
NET	D64 (h)	F72 (h)	D75 (h)	A77 (h)	N78 (h)	W80 (h)	C86 (h)	F94 (h)	Y98 (h)	L100 (h)	F101 (h)	A105 (h)	E113 (h)	G117 (h)

	2.63	2.65	3.46	3.48	3.49	3.50	3.52	3.53	3.54	4.25	4.54	5.27	5.30	5.36
DAT	R125 (h)	G127 (h)	V152 (h)	F154 (r)	F155 (h)	Y156 (h)	V158 (h)	I159 (h)	I160 (h)	E218 (h)	V247 (h)	K264 (h)	W267 (h)	P272 (r)
SERT	R144 (h)	G146 (h)	I172 (r)	S174 (h)	Y175 (h)	Y176 (r)	T178 (h)	I179 (h)	M180 (h)	R234 (h)	F263 (h)	K279 (h)	W282 (h)	P288 (h)
NET	R121 (h)	G123 (h)	V148 (h)	F150 (h)	Y151 (h)	Y152 (h)	V154 (h)	I155 (h)	I156 (h)	E215 (h)	V244 (h)	K261 (h)	W264 (h)	P270 (h)

	5.37	6.40	6.44	6.46	6.52	6.68	7.30	7.42	7.44	7.45	7.47	8.23	8.29	8.40
DAT	Y274 (h)	E307 (h)	W311 (h)	D313 (h)	C319 (h)	Y335 (h)	D345 (h)	S356 (r)	S359 (h)	S359 (r)	F361 (r)	D385 (h)	F390 (r)	P402 (h)
SERT	Y289 (h)	E322 (h)	W326 (h)	D328 (h)	F334 (h)	Y350 (h)	D360 (h)	S372 (r)	V374 (h)	S375 (r)	F377 (h)	A401 (h)	F407 (h)	P418 (h)
NET	Y271 (h)	E304 (h)	W308 (h)	D310 (h)	F316 (h)	Y332 (h)	D342 (h)	S354 (h)	V356 (h)	S357 (h)	F359 (h)	E382 (h)	F388 (h)	S399 (h)

	8.41	8.45	8.59	9.38	9.47	10.26	10.28	10.41	10.46	10.47	11.45	11.49	11.50	12.58
DAT	L403 (h)	W406 (r)	D421 (h)	T455 (r)	T464 (r)	D476 (h)	F478 (h)	E490 (r)	F513 (b)	W496 (r)	W523 (r)	S528 (h)	P529 (h)	M569 (h)
SERT	A419 (h)	F423 (h)	D437 (h)	C473 (h)	T482 (h)	E493 (h)	F495 (b)	E508 (h)	S513 (h)	W514 (h)	W541 (h)	S545 (r)	P546 (h)	F586 (h)
NET	G400 (h)	W404 (h)	D418 (h)	T453 (h)	T462 (h)	D473 (h)	F475 (h)	E488 (h)	S493 (h)	W494 (h)	W521 (h)	S525 (h)	P526 (h)	M566 (h)

*DAT indicates dopamine transporter; NET, norepinephrine transporter; and SERT, serotonin transporter. Residues are grouped by TM domain and color-coded to match the transmembrane (TM) domain in Figure 1. Individual residues are named by their one-letter amino acid code and position in the polypeptide chain of that transporter, parenthetically followed by the species employed in mutagenesis studies (eg, "D68 (h)" refers to the aspartic acid residue of the human DAT). Residues at the analogous position in the neurotransmitter:sodium symporter (NSS) family sequence alignment are collectively named using the indexing system described in the Introduction (from Goldberg et al[17]). Thus, hDAT D68, hSERT D87, and hNET D64 are found at Position 34 in TM 1. In many cases, only one monoamine transporter was mutated at the indicated position; the analogous residues in the other 2 transporters are shown as points of reference.

$D98_{1.45}$ yielded a functional SERT.[23] From experiments employing shortened tryptamine analogs, formation of a $D98_{1.45}$-serotonin salt bridge was judged to be probable.[23] Finally, the premise that a positively charged tropane nitrogen atom of cocaine or its analogs is critical for inhibition of dopamine uptake at the DAT[24] is in question,[25,26] as is formation of a $D_{1.45}$-cocaine salt bridge.[22]

Primarily to search for DAT TM residues capable of directly interacting with the positively charged moieties of substrates and inhibitors, conserved acidic and tryptophan hDAT residues were separately mutated and the mutant transporters characterized, including the TM 1 residues $D68_{1.34}$ and $W84_{1.50}$.[27] The conservative asparagine-for-aspartate mutant $D68_{1.34}N$, presumably positioned at the intracellular interface of TM 1[17,27] (Figure 1), displayed 3- to 4-fold losses in affinity for the cocaine analog WIN 35,428 and in cocaine DUIP. The mutation did not appreciably affect recognition of the classic DAT inhibitor GBR-12909 or most of the hydroxypiperidine GBR-like analogs tested; however, one such analog, (+)-*R,R*-D-84, sustained a 17-fold affinity loss. Of interest, this potential anticocaine therapeutic differs from one of the analogs unaffected by the mutation only in the position of a hydroxyl group, suggesting a direct interaction between $D68_{1.34}$ and the (+)-*R,R*-D-84 hydroxyl moiety.[28]

Human ($W84_{1.50}L$) and rat ($W84_{1.50}A$) DAT substitutions of the $W_{1.50}$ residue actually increased WIN 35,428 affinity and cocaine DUIP; dopamine K_m (Michaelis constant) values were unaffected.[27,29] $W84_{1.50}$ may contribute to maintaining an intracellular-facing DAT conformation,[27] and Na^+-dependent conformational changes required for DAT function were impaired in hDAT $W84_{1.50}L$.[18] This mutant also displayed Na^+ sensitivity differences between cocaine and the diphenylmethoxy-bearing compounds benztropine and GBR-12,909. Taken with the aforementioned rDAT $D79_{1.45}E$ results, the hDAT $W84_{1.50}L$ findings suggest that TM 1 residues may provide discrimination between diphenylmethoxy-bearing compounds and classic inhibitors such as cocaine, WIN 35,428, and mazindol. Moreover, of several endogenous hDAT cysteine residues surveyed for accessibility to the methanethiosulfonate alkylating agent MTSET, the benztropine-induced DAT alkylation pattern diverged from those of cocaine, WIN 35,428, mazindol, and dopamine only at $C90_{1.56}$, a residue immediately extracellular to TM 1.[30] It is unclear whether these TM 1-associated inhibitor selectivities are solely due to DAT conformational differences or are indicative of TM 1 contributions to inhibitor binding sites.

SERT mutagenesis findings are consistent with direct interactions between TM 1 residues and uptake inhibitors or substrates. In addition to compensatory effects on serotonin analog affinities, the $D98_{1.45}E$ mutant sustained serotonin uptake inhibition potency (SUIP) losses for cocaine, imipramine, and citalopram but not paroxetine or mazindol.[23] Human/*Drosophila* SERT chimera studies led

to identification of hSERT $Y95_{1.42}$ ($F90_{1.42}$ in dSERT) as solely accounting for species differences in recognition of tryptamine analogs.[31] Using SERTs from the same 2 species, the hSERT $Y95_{1.42}$ side chain was previously postulated to sterically clash with the hydroxyl group of mazindol, while citalopram experienced no such hindrance.[32] The authors note that hSERT $Y95_{1.42}$ is one α-helical turn below $D98_{1.45}$ and therefore on the same face of TM 1, possibly facilitating coordination of the 2 residues in binding substrates and inhibitors.[31] Scanning cysteine accessibility mutagenesis (SCAM)[33] of all putative hSERT TM 1 residues revealed that $D98_{1.45}C$, $G100_{1.47}C$, and $N101_{1.48}C$ were protected from MTSET inactivation by serotonin; $G100_{1.47}C$ and $N101_{1.48}C$ were similarly protected by cocaine.[34] The latter TM 1 residues are above (extracellular to) $Y95_{1.42}$ and $D98_{1.45}$ and may indeed be shielded from alkylation by the ligand; still, the ligand may have instead induced a conformational shift in SERT that in turn altered MTSET accessibility to the cysteine mutants. Nonsubstrates including cocaine are capable of altering DAT conformations in a manner sensitive to both the SCAM assay[30,35] and to changes in DAT vulnerability to proteases.[36]

Side chains at the position analogous to hSERT $Y95_{1.42}$ in other NSS members have been associated with substrate or inhibitor recognition. Alanine substitution of rDAT $F76_{1.42}$ decreased WIN 35,428 binding 4-fold without decreasing apparent surface expression. Dopamine potency in displacing [^{3}H]-WIN 35,428 at this mutant, however, increased ~300-fold, suggesting a significant perturbation in DAT structure or in available DAT conformations.[37] The hSERT tyrosine residue is conserved in only 1 of the 4 GABA transporters, with a glutamate side chain occupying the position in GAT-2, -3 and -4. Characterization of GAT-4 $E61_{1.42}$ mutants suggested that this side chain contributes to substrate binding.[38] Findings from TM 1 SCAM of GAT-1 were similar to hSERT in that the GAT-1 TM 1 also appears to contribute to the substrate permeation pathway.[34,39]

TM 2

Alanine replacement of rDAT $F98_{2.36}$, a residue putatively at the TM 2 extracellular interface, decreased WIN 35,428 binding 6-fold. The K_m value for dopamine uptake was unaffected, but dopamine turnover rate was greatly diminished.[37] $F98_{2.36}$ is largely conserved in the NSS family and perhaps less likely to directly contact specific uptake inhibitors. A mouse/*Drosophila* DAT chimera study led to identification of mDAT $F105_{2.43}$ as the residue chiefly responsible for the 10-fold higher DUIP of cocaine at the mDAT; this position is occupied by methionine in the dDAT. Of several mutations tested, only the presence of an aromatic side chain at mDAT position $105_{2.43}$ retained wildtype-like DUIPs for cocaine. It was not determined whether the effect of nonaromatic substitution of mDAT $F105_{2.43}$ on cocaine DUIP was direct or indirect.[40] Curiously, WIN 35,428 affinity at rDAT $F105_{2.43}A$ decreased by only 2-fold relative to wildtype rDAT.[37] Methionine is also found at this position in some SERTs, but a phenylalanine residue one α-helical turn above, $F127_{2.40}$ in hSERT ($I108_{2.40}$ in mDAT), may play the role of mDAT $F105_{2.43}$ with respect to cocaine potency.[40] SCAM analysis of rSERT TM 2, however, did not identify residues that directly affected substrate binding or were accessible to alkylating agents.[41] Most recently, random mutagenesis of mDAT TM 2 residues in the vicinity of $F105_{2.43}$ generated the triple mutant $L104_{2.42}V/F105_{2.43}C/A109_{2.47}V$, which suffered 69- and 47-fold DUIP losses for cocaine and methylphenidate. The DUIPs for the substrates amphetamine and methamphetamine at the triple mutant were not significantly different from those at the wildtype mDAT.[42]

A glutamate residue at Position 2.55 ($E_{2.55}$) is absolutely conserved within the NSS family. This residue is thought to be at the cytoplasmic interface of TM 2 but could instead be the initial residue of ICL 1. If in the TM domain, $E_{2.55}$ would be only the third acidic TM residue and would likely be situated in a hydrophilic zone such as a ligand/ion pore. Human DAT and NET and rat GAT-1 proteins have been mutated at this position. The hDAT $E117_{2.55}Q$ mutation was not localized to the plasma membrane and was not characterized further.[27] Replacement of hNET $E113_{2.55}$ with alanine or aspartic acid compromised cell surface expression and eliminated norepinephrine uptake; substitution with glutamine was well tolerated. Affinity for the NET-selective inhibitor nisoxetine was reduced 10-fold at hNET $E113_{2.55}A$, with little or no change at $E113_{2.55}D$ and $E113_{2.55}Q$. Apparent affinities for substrates, measured by displacement of [^{3}H]-nisoxetine, were drastically reduced only at hNET $E113_{2.55}D$; cocaine and desipramine affinities were altered by less than 3-fold for all mutants. Thus, a 1-carbon shorter side chain at this position somehow profoundly disturbs substrate binding.[43] Glutamine substitution of the analogous rGAT-1 residue ($E101_{2.55}Q$) disrupted Na^+ binding, in turn disrupting GABA transport[44]; the hNET $E113_{2.55}Q$ mutant did not suffer the same functional consequences.[43]

TM 3

Human/bovine DAT chimeras identified a 54-residue segment encompassing TM 3 as especially critical for dopamine uptake and WIN 35,428 binding.[45] Remarkably, replacement of the bDAT TM 3 residue $I152_{3.46}$ with its conservative valine counterpart in the hDAT was found to almost single-handedly confer the superior substrate transport and WIN 35,428 binding characteristics of the hDAT.[46] Two positions away, $F154_{3.48}$ also appears to be relevant to

cocaine recognition, as the rDAT $F154_{3.48}A$ mutation decreased cocaine affinity 10-fold without appreciably affecting substrate uptake.[47] $V152_{3.46}$ and $F154_{3.48}$ should be on opposite faces of DAT TM 3, meaning that both cannot directly contact the ligand. SCAM analysis of TM 3 of rSERT indicated that $I172_{3.46}$, the residue analogous to hDAT $V152_{3.46}$, is on the helical face accessible to ligands and external agents.[48] Moreover, $I172_{3.46}$ and $Y176_{3.50}$ of rSERT are in or near the binding sites for serotonin and cocaine.[48,49] Assuming that TM 3s of the DAT and SERT have similar orientations in the plasma membrane, $V152_{3.46}$ would be expected to face the ligand pore and $F154_{3.48}$ would face the lipid bilayer. $F155_{3.49}$ of DAT could still face the ligand pore, a residue conserved among DATs but replaced by tyrosine in SERTs and NETs. The rDAT $F155_{3.49}A$ mutant sustained a profound loss in apparent affinity for dopamine, but only a mild decrease in WIN 35,428 affinity.[37]

Two helical turns above $I172_{3.46}$, substrate transport but not cocaine binding by SERT $I179_{3.53}C$ was inactivated in the presence of MTSET. Neither substrate nor cocaine protected the mutant from the alkylating agent.[48] MTSET alkylation of the analogous NET mutant ($I155_{3.53}C$) was inhibited and enhanced by dopamine and cocaine, respectively. Substrate protection of this mutant was Na^+ and temperature dependent, suggesting a conformationally-sensitive protection mechanism as opposed to direct substrate occlusion of $I155_{3.53}C$ access.[49] In contrast to the SERT and NET, the analogous DAT mutant ($I159_{3.53}C$) was essentially insensitive to MTSET, in the presence or absence of dopamine or cocaine.[50] Thus, the $I_{3.53}$ residue in the monoamine transporters is less likely to directly contribute to ligand binding but has been proposed to contribute toward an external gate for the substrate permeation pathway.[49] Consistent with this idea, alanine mutation of the flanking $T178_{3.52}$ hSERT residue greatly accelerated serotonin translocation, apparently by modifying the equilibrium of SERT conformations.[51] Through human/bovine SERT species scanning and reverse mutations, $M180_{3.54}$, the other hSERT residue flanking $I179_{3.53}$, was found to substantially contribute to hSERT's higher SUIPs for the antidepressants citalopram, paroxetine, fluoxetine, and imipramine. A citalopram structure-activity series suggested that the heterocyclic nucleus of the drug interacts with $M180_{3.54}$.[52] This side chain also contributes to an allosteric citalopram binding site of the hSERT.[53]

TM 4

The pharmacologic profile of the hSERT $F263_{4.54}C$ mutant very closely resembles that of hSERT $T178_{3.52}A$ described above. Of 24 SERT mutants tested, only these 2 mutants displayed notable (~5-fold) K_m and V_{max} increases. The effect was amplified by combining the mutations, with K_m and V_{max} increases of 50- and 10-fold, respectively. A synergistic effect was also seen with respect to binding of the cocaine analog RTI-55, the double mutant sustaining a 60-fold affinity loss without a significant B_{max} change. Both individual mutants were insensitive to MTS reagent effects. The $F263_{4.54}$ residue alone appeared relatively unimportant with respect to the SUIPs of 5 inhibitors tested.[51]

TM 5

The $W267_{5.30}L$ hDAT mutation decreased cocaine DUIP by only 3-fold; uptake kinetics suggest that $W267_{5.30}$ contributes to an outward- (extracellular-) facing DAT conformation.[27] This residue is expected to border the cytoplasm. For hNET, the highly conserved $Y271_{5.37}$ residue was substituted with alanine, phenylalanine, or histidine; only the alanine mutant altered (decreased) the norepinephrine uptake inhibition potency (NUIP) of cocaine by 3-fold, with more modest effects on nisoxetine and desipramine NUIPs. Apparent affinities for norepinephrine and MPP^+ increased 3- to 4-fold at hNET $Y271_{5.37}A$.[54] Glycine replacement of rDAT $P272_{5.36}$ modestly reduced dopamine uptake but decreased WIN 35,428 binding affinity 10-fold without a reduction in B_{max} value. DUIPs for cocaine, mazindol, BTCP (1-[1-(2-benzo[b]thiopheneyl)cyclohexyl]piperidine hydrochloride), and trihexyphenidyl decreased by over 100-fold.[55] A subsequent study yielded very similar findings regarding dopamine uptake kinetics and WIN 35,428 binding with the same mutant and additionally characterized the rDAT $P272_{5.36}A$ mutation. The alanine mutant decreased WIN 35,428 affinity only 4-fold relative to wildtype rDAT.[56] Alanine replacement of the analogous hNET residue $P270_{5.36}$ yielded undetectable specific binding of nisoxetine and 11-, 3-, and 3-fold decreases in the NUIPs of nisoxetine, desipramine, and cocaine, respectively. Of 10 hNET proline residues mutated, only $P270_{5.36}A$ decreased recognition of uptake inhibitors by 3-fold or more.[57]

The number of TM proline residues is observed to be disproportionately large in transport proteins relative to other integral membrane proteins, yet it is unclear how such TM proline residues affect transporter protein structure and function.[58,59] In general, proline residues, and to a lesser extent glycine residues, disrupt α-helices, whereas alanine residues promote α-helix formation.[60] The extent of the α-helical "kink" induced by proline, however, is dependent on its environment,[61] and especially on neighboring residues.[62,63] Proline residues may serve a structural role, determining protein infrastructure by influencing helix-helix packing. Functional roles for TM proline residues include providing hinges that facilitate signal transduction, mediating conformational changes via *cis-trans* isomerization of the bond linking the proline to the preceding residue of the polypeptide, and providing a geometry that allows

neighboring amide carbonyl oxygen atoms of the polypeptide to serve as cation binding sites.[64-66] The latter functional role is most likely for the monoamine transporters. Thus, $P272_{5.36}$ may provide a direct ligand binding site, a key Na^+ binding site that modulates transport or substrate or inhibitor recognition, or simply a kink necessary to the ligand or ion binding pocket.

TM 6

Leucine replacement of hDAT $W311_{6.44}$, putatively at the extracellular interface, decreased WIN 35,428 affinity 10-fold and cocaine DUIP over 3-fold; dopamine displacement of WIN 35,428 decreased by over 100-fold.[27] In contrast, alanine replacement of the rDAT counterpart ($W310_{6.44}A$) actually increased WIN 35,428 affinity 4-fold, and dopamine displacement of the cocaine analog was over 200 times more effective.[29] It should be noted that the rDAT binding was conducted at 4°C, compared with 37°C in the hDAT study.[27] Two residues away, the hDAT $D313_{6.46}N$ mutant did not notably affect WIN 35,428 or cocaine binding under normal assay conditions, and dopamine affinity was decreased.[27] While not believed to be part of the substrate or inhibitor binding sites, $D313_{6.46}$ may nevertheless regulate access to external dopamine in a Na^+-dependent fashion. This residue and $W84_{1.50}$ are involved in cation interactions, and control in part the ability of Na^+ to drive the DAT between inward- and outward-facing conformations, in turn influencing dopamine access and Na^+-dependent cocaine affinity.[67,68]

Searching for tricyclic antidepressant binding sites based on findings from chimeric studies,[69,70] nonconserved hNET residues from a region spanning TM 5 to TM 8 were replaced with the hDAT counterparts at each of 24 positions. The TM 6 residue $F316_{6.52}$ was the most important of the 24 for desipramine actions, as the hNET $F316_{6.52}C$ mutation reduced its DUIP 6-fold. Nortriptyline DUIP decreased 8-fold, but cocaine potency doubled. The reverse mutation in hDAT ($C319_{6.52}F$) increased DUIPs for these tricyclics, albeit less than 3-fold. $F316_{6.52}$ is conserved among all tricyclic-sensitive NSS members, mammalian or otherwise.[71] It remains to be elucidated if $F316_{6.52}$ directly interacts with tricyclic drugs.

TM 7

Simultaneous mutation of the rDAT $S356_{7.42}$ and $S359_{7.45}$ residues to glycine or alanine resulted in mild-to-insignificant reductions in WIN 35,428 binding affinity and significant but modest decreases in dopamine uptake. These residues are postulated to form hydrogen bonding interactions with the catechol hydroxyl groups of dopamine.[19] Again, this model was borrowed from that of Strader and colleagues, who demonstrated an association between β-adrenergic receptor TM serine residues and agonist hydroxyl groups.[72] The fact that a serine side chain is found at position 7.42 throughout the NSS family, including transporters for noncatechol substrates, argues against this model. In contrast, a serine is found at position 7.45 for the monoamine transporters and few other NSS members. The analogous hNET serine residues ($S354_{7.42}$ and $S357_{7.45}$) were singly and jointly switched to alanine. Affinity for nisoxetine was reduced 70-fold at hNET $S354_{7.42}A$, while affinity was unchanged at the $S357_{7.45}A$ mutant. Affinities for dopamine and *m*- and *p*-tyramine, dopamine analogs lacking either of the catechol hydroxyl groups, were reduced at hNET $S354_{7.42}A$, but not in a manner that suggested a direct interaction with a catechol hydroxyl. Recognition of the 3 substrates by hNET $S357_{7.45}A$ was indistinguishable from wildtype hNET. The authors concluded that formation of a hydrogen bond between these serine residues and substrates was unlikely.[73] Alanine mutation of either analogous rSERT serine residue ($S372_{7.42}$ and $S375_{7.45}$) only slightly diminished serotonin transport; inhibitor affinities were not investigated. Random mutagenesis of TM 7 revealed that most of the residues important to transporter function or Na^+ dependence were expected to share the same α-helical face, the face shared by $S372_{7.42}$ and $S375_{7.45}$.[74] TM 7 SCAM analysis indicated that this helical face is not directed toward a water-accessible pore. The TM 7 face containing $S372_{7.42}$ and $S375_{7.45}$ is probably involved in helix-helix interactions with another SERT TM domain, a role common to TM serine residues. In this way, TM 7 may transmit ion-driven conformational shifts in the protein.[75] There is no evidence that $S372_{7.42}$ and $S375_{7.45}$ contribute directly to inhibitor binding sites.

The presence of a third serine residue between $S372_{7.42}$ and $S375_{7.45}$ accounts in part for the lower tricyclic antidepressant DUIPs at the DAT relative to the hNET. Mutation of hNET $V356_{7.44}$ to the analogous hDAT serine residue decreased DUIPs for nortriptyline and desipramine by 10- and 4-fold, respectively. The DUIP for cocaine was not altered appreciably. The reverse mutation in hDAT ($S359_{7.44}V$) in turn increased DUIPs for nortriptyline and desipramine by 5- and 10-fold, respectively.[71]

Finally, alanine replacement of rDAT $F361_{7.47}$ decreased WIN 35,428 binding affinity by an order of magnitude without affecting dopamine uptake kinetics.[37] Positioned at the TM 7 midpoint, this side chain is largely conserved in the NSS family.

TM 8

DUIPs for desipramine and nortriptyline were respectively reduced 3- and 6-fold by substituting hNET $G400_{8.41}$ with leucine, the analogous hDAT residue. The reciprocal

mutation in hDAT ($L403_{8.41}G$), however, did not affect the DUIPs for these drugs. The hNET triple mutant containing the aforementioned TM 6 and TM 7 substitutions ($F316_{6.52}C$/$V356_{7.44}S$/$G400_{8.41}L$) reduced desipramine and nortriptyline DUIPs 35- and 9-fold, with little change in dopamine uptake kinetics. Replacement of hNET $S399_{8.40}$ with the proline side chain found at hDAT position 8.40 did not affect inhibitor actions alone, but the double mutant $S399_{8.40}P$/$G400_{8.41}L$ decreased respective DUIPs for desipramine and nortriptyline by 1000- and 80-fold without altering cocaine DUIP or uptake kinetics. The dose-response curves for this double mutant were biphasic, and in fact correlate well with the high and low affinity IC_{50} values for tricyclics at hNET and hDAT, respectively.[71] Mutation of this region of TM 8 may alter the balance of inhibitor binding NET conformations.

For the rDAT, alanine substitution of $W406A_{8.45}$ decreased WIN 35,428 binding affinity by 3-fold; V_{max} and K_m values for dopamine uptake decreased 10-fold and 6-fold, respectively.[29] This residue is not far from the extracellular interface and is largely conserved in the NSS family, although a phenylalanine side chain is found at the same position in the SERT.

TM 9

TM 9 is underrepresented as a target for NSS structure-function studies on inhibitor recognition. Of the handful of mutants characterized with respect to uptake inhibitors, only 2 exhibited binding affinity or uptake inhibition potency shifts of 3-fold or more. WIN 35,428 binding affinities for the rDAT $T455_{9.38}A$ and $T464_{9.47}A$ mutants were, respectively, 20- and 7-fold lower than at the wildtype rDAT. Dopamine turnover was unchanged at rDAT $T455_{9.38}A$ and increased 5-fold at rDAT $T464_{9.47}A$.[76,77]

TM 10

TM 10 SERT residues at Positions 10.28 and 10.46 are reported to collaborate with the $M180_{3.54}$ side chain in accounting for the higher SUIPs of selected antidepressants at the hSERT compared with the bSERT. Gain-of-function bSERT mutants ($F495_{10.28}Y$ and $F513_{10.46}S$) and reverse hSERT mutants ($Y495_{10.28}F$ and $S513_{10.46}F$) confirmed that like hSERT $M180_{3.54}$, the presence of the hSERT side chain at each TM 10 position conferred higher SUIPs for citalopram and paroxetine. $S513_{10.46}$ and $M180_{3.54}$ were most important for fluoxetine and imipramine SUIPs. Because the SUIP gain-of-function was similar regardless of whether serine or alanine was substituted for bSERT $F513_{10.46}$, the phenylalanine side chain was postulated to confer steric hindrance in binding of these antidepressant uptake blockers. These TM 10 residues should lie on the same α-helical face and may line an antagonist binding pocket.[52]

For the rDAT, alanine mutation of $W496_{10.47}$ decreased WIN 35,428 binding affinity 6-fold, with minor effects on dopamine uptake kinetics.[29] This highly, but not absolutely, conserved residue should be within a helical turn of the cytoplasm. Finally, it should be noted that a glutamic acid residue is projected to be well within TM 10 ($E_{10.41}$), and a glutamic acid side chain is found at this position in over 50% of the NSS members, including the monoamine transporters. $E_{10.41}$ is one of only 2 monoamine transporter side chains with a full negative charge that almost certainly resides in the TM domains (the other being $D_{1.45}$); thus, $E_{10.41}$ would have to line a hydrophilic enclave such as a ligand or ion binding pore. Surprisingly, this residue has received little attention in structure-function studies. Other than a textual communication that the rDAT $E490_{10.41}A$ mutant was deficient in dopamine uptake and in registering an immunochemical signal[76]; no data are published on mutations at this position. A more conservative substitution (eg, $E_{10.41}Q$) may therefore be useful in searching for NSS protein contributors to ligand and ion recognition.

TM 11

Just as mutation of TM 10 residues compromised SUIPs of selected antidepressants, the rSERT $S545_{11.49}A$ mutant displayed a 7-fold SUIP decrease for citalopram, although high affinity binding of the drug was unchanged. Conversely, high affinity binding of imipramine was decreased 5-fold at the mutant, with a more subtle SUIP decrease. Of interest, this mutation allowed wildtype-like transport function when Li^+ was substituted for Na^+. An ion gating role for rSERT $S545_{11.49}$ may modulate conformational changes important for recognition of these antidepressants.[78] $S545_{11.49}$ is conserved among the monoamine transporters and highly conserved within the NSS family, occasionally replaced by threonine. Replacing the very highly conserved $P_{11.50}$ residue with alanine in the hNET actually increased (by 3-fold) NUIPs for nisoxetine, desipramine, and cocaine.[57]

TM 11 mutants displaying significant alterations in inhibitor affinities or uptake inhibition potencies are otherwise in short supply. WIN 35,428 affinity was decreased 4-fold at rDAT $W523_{11.45}A$. Dopamine uptake V_{max} was reduced 13-fold despite a wildtype-like B_{max} value.[29]

TM 12

Chimeric and point mutagenesis revealed that hSERT $F586_{12.58}$ almost solely accounts for the higher SUIPs of the tricyclic antidepressants imipramine, desipramine, and nortriptyline at hSERT relative to rSERT.[79,80] This hSERT phenylalanine residue is replaced by a valine side chain in other SERT species, and methionine in the DAT and NET. The relationship held for both gain-of-function (rSERT $V_{12.58}F$)

and reverse (hSERT $F_{12.58}V$) mutants. SUIPs for cocaine, amphetamine, and nontricyclic antidepressants were not affected by this mutation. Curiously, the rSERT $V_{12.58}D$ mutation provided the same gain-of-function, but the rSERT $V_{12.58}Y$ mutation did not. Cocaine SUIP was also augmented by the $V_{12.58}D$ substitution.[80] That $V_{12.58}$ substitution with phenylalanine and aspartate side chains yields similar antidepressant potency increases suggests that different intramolecular interactions are formed that influence either the inhibitor binding sites or the prevalence of their preferred rSERT conformations. Introduction of a phenylalanine side chain at the analogous hNET position ($M566_{12.58}F$) did not alter DUIPs for the tricyclic compounds, suggesting a nonidentical antidepressant binding site for this transporter.[79,80]

Extratransmembranous Regions

Extracellular Loops

While it may be feasible to design a monoamine transporter-selective uptake inhibitor that reversibly binds to one or more extracellular loops (ECLs) and controls substrate access, binding sites for the classic, physiologically relevant inhibitors are believed to reside within the TM domains. Of the many ECL mutations created or found as polymorphisms within NSS family members, few have an appreciable effect on recognition of uptake inhibitors, and those that do are expected to alter transporter conformations as opposed to direct modification of inhibitor binding sites.[71,81]

Many mutations of putatively extracellular and intracellular residues have been generated for the purpose of detecting conformational shifts in, or mapping tertiary structure of, NSS family members. A few of these mutations were demonstrated to affect uptake inhibitor recognition. The hDAT $C90_{1.56}A$ mutant was prepared toward generating a DAT species lacking endogenous methanethiosulfonate-reactive cysteine residues.[35] This mutation did not alter affinity for WIN 35,428, but selectively compromised the DUIP of benztropine; DUIPs of cocaine, WIN 35,428, and mazindol were similar to wildtype hDAT. This very highly conserved ECL 1 residue is not expected to directly contact benztropine, but rather to differentially contribute toward stabilizing benztropine- versus cocaine-preferring DAT conformations.[30,35] Gether and colleagues have mutated many extratransmembranous hDAT residues in elucidating the endogenous Zn^{2+} binding site of the DAT, and in creating new Zn^{2+} sites toward mapping TM domain proximities.[50,82,83] In the course of this work, the extracellular hDAT mutants $E218_{4.25}Q$ (ECL 2), $E307_{6.40}Q$ (ECL 3), and $D385_{8.23}N$ (ECL 4) were found to sustain 4- to 5-fold losses in WIN 35,428 affinity.[83]

Regarding other ECL mutations, WIN 35,428 affinity decreased 5-fold as a result of the ECL 4 rDAT $F390_{8.29}A$ mutation; dopamine uptake was virtually eliminated.[37] Asparagine substitution of hDAT $D476_{10.26}$, a residue at the ECL 5/TM 10 interface, decreased WIN 35,428 affinity 4-fold, cocaine DUIP 3-fold, and apparent affinity (measured by K_m value) for dopamine 7-fold.[27] For the above ECL loop mutants, the inhibitor binding affinity and uptake inhibition potency losses are likely due to mutation-induced conformational changes; however, alterations in the actual inhibitor binding sites have not been ruled out.

Intracellular Segments

The N- and C-terminal tails of NSS family members do not appear to contain residues that directly contact inhibitors of substrate uptake. Nevertheless, mutagenesis studies indicate that selected N-terminal residues are physiologically relevant to the function of at least one inhibitor of monoamine uptake—amphetamine. As a structural analog of dopamine, the binding site for amphetamine, a monoamine transporter substrate, is expected to overlap with that of the neurotransmitter. The classical model posits that amphetamine uptake increases the percentage of inward-facing substrate binding sites of the transporter, facilitating reverse transport ("efflux") of cytoplasmic monoamine neurotransmitter into the synapse.[84] Recently, alanine substitution of the 5 hDAT N-terminal serine residues was found to almost eliminate amphetamine-mediated dopamine efflux without affecting dopamine uptake. Efflux was restored by substituting aspartic acid, a phosphorylation mimic, at 2 of the 5 positions.[85] The same N-terminal region is also required for PKC-mediated phosphorylation of the hDAT.[86] The necessity of phosphorylation for amphetamine-induced hDAT reverse transport but not for dopamine uptake suggests a problem with the classical "alternating access" model, and in fact, amphetamine actions at monoamine transporters may be better explained by an oligomeric DAT complex with separate moieties for forward and reverse transport.[87]

The remaining ICL mutants discussed below are not PKC substrates but are also not expected to make contact with the uptake inhibitor. In exploring the $G_{2.59}XXXR_{2.63}XG_{2.65}$ motif in the hNET ICL 1, the $G117_{2.59}A$ mutation decreased the NUIPs of desipramine and nisoxetine 5-fold without affecting that for cocaine. In contrast, alanine replacement of $G123_{2.65}$ did not alter the antidepressant NUIPs but decreased cocaine NUIP almost 3-fold. The results are consistent with the existence of nonidentical hNET binding sites for antidepressants and psychostimulants.[88] Mutation of these absolutely conserved residues probably disturbs inhibitor site infrastructure or the NET conformational equilibrium.

The intracellular hDAT mutations $K264_{5.27}A$ (ICL 2), $Y335_{6.68}A$ (ICL 3), $D345_{7.30}A$ (ICL 3), and $D421_{8.59}A$ (ICL 4) decreased WIN 35,428 affinity by 5-, 60-, 5-, and 9-fold, respectively. Cocaine DUIPs were reduced at the $K264_{5.27}A$,

$Y335_{6.68}A$, and $D345_{7.30}A$ mutants by 5-, 70-, and 7-fold; for the same mutants, GBR-12,909 DUIPs were reduced 3-, 10-, and 3-fold.[50] A previous study of hDAT $Y335_{6.68}A$ revealed 10- to 150-fold DUIP decreases for WIN 35,428, cocaine, GBR-12,909, mazindol, and the cocaine analog RTI-55, but a 4-fold increase in amphetamine DUIP. Addition of micromolar Zn^{2+} concentrations substantially mitigated the DUIP losses.[89] As expected, the deficits appeared to be due to infrastructural changes; such changes may be shifting the equilibrium between DAT conformations.[50] The TM 3 $I159_{3.53}C$ mutation was introduced into these hDAT mutants, and resultant constructs were compared with hDAT $I159_{3.53}C$ for methanethiosulfonate reagent cross-linking in the presence or absence of Zn^{2+} or cocaine. The fact that cocaine decreased MTSET accessibility of the $I159_{3.53}C$ side chain in an otherwise wildtype hDAT protein but increased accessibility of this cysteine upon addition of the $K264_{5.27}A$, $Y335_{6.68}A$, or $D345_{7.30}A$ substitutions suggests that these 3 mutants employed a different cocaine-bound DAT conformation relative to wildtype hDAT.[50] Of interest, MTSET accessibility for hNET $I155_{3.53}C$ also increased upon addition of cocaine,[49] suggesting that the cocaine-bound state of hNET differs from that of hDAT.[50]

A recent in-depth study of hDAT $D345_{7.30}N$ indicated that like hDAT $Y335_{6.68}A$, $D345_{7.30}N$ is an ICL 3 mutation that increased DUIPs for amphetamine and other substrates. In addition, the usual uptake inhibition role of Zn^{2+} was actually reversed at these 2 mutants so that dopamine uptake was potentiated.[90] Unlike hDAT $Y335_{6.68}A$, the $D345_{7.30}N$ mutant did not affect inhibitor DUIPs, except for a 2-fold decrease in cocaine potency. The very low K_m and V_{max} values for dopamine uptake, the Zn^{2+} potentiation of uptake, and the lack of amphetamine-mediated dopamine efflux suggest that this mutation appears to render a preference for an inward-facing DAT conformation. A fascinating aspect of this mutant is that while most classical inhibitors potently block substrate uptake at hDAT $D345_{7.30}N$, high affinity specific binding of radiolabeled versions of the same inhibitors was undetectable under the same conditions. Detection of specific radioligand binding was also a function of whether the transfected cells were intact monolayers or membrane preparations at the time of assay.[90] This phenomenon is infrequently documented but has been reported in other DAT mutants.[21,91] One explanation for this curiosity is that the small percentage of remaining outward-facing DAT conformation(s) for hDAT $D345_{7.30}N$ is insufficient for detection of binding of the inhibitor radiotracer, but adequate for higher levels of inhibitors and substrates to bind and drive the conformational cycling forward such that dopamine uptake (and its inhibition) is detected.[90] An alternative explanation is that DAT populations exist, possibly composed of multimeric DAT complexes[92-94] or DAT associated with intracellular factors,[95-98] and that the $D345_{7.30}N$ mutation eliminates the population responsible for high affinity radioligand binding without affecting the population(s) most critical for substrate uptake and its inhibition.[21,22,91] Regardless of the explanation, a lack of correlation between an inhibitor's binding affinity and substrate uptake inhibition potency values at a given wildtype or mutant transporter protein is well documented.[21,22,88,90,99,100]

CONCLUSIONS

While elucidation of plasma membrane monoamine transporter inhibitor binding sites has progressed steadily, the lack of a crystal structure for any member of the NSS family has been a hindrance. Indeed, no high resolution structure is available for any mammalian transporter protein close to the NSS family on the phylogenetic tree, although recent DAT modeling based on the crystal structure of the bacterial 12 TM Na^+/H^+ transporter NhaA has yielded predictions largely consistent with the mutagenesis data regarding the role of specific DAT residues.[101-104] The very recently published crystal structure of a bacterial leucine transporter ($LeuT_{Aa}$), a protein with mild but definite amino acid sequence similarity to the NSS family, is also suggested to be a template for NSS protein modeling.[105] Binding pockets for the leucine substrate and one of 2 Na^+ atoms are created primarily by the middle portions of TM 1 and TM 6. Of interest, unwinding of the center of these 2 TM domains exposes main chain carbonyl oxygen and nitrogen atoms that H-bond to substrate and Na^+; no $LeuT_{Aa}$ side chains bearing a formal charge interact with the substrate. The unwound regions of TM 1 and TM 6 are also postulated to serve as hinges involved in interconversion of outward- and inward-facing transporter conformations. Still, it cannot be assumed at this point that the NSS proteins employ the same transport mechanism, or even that NSS TM domains are arrayed identically to $LeuT_{Aa}$ (eg, the significantly shorter ICL 1 segment for NSS proteins may preclude the TM 2-TM 3 spatial arrangement found in $LeuT_{Aa}$).

No direct contacts between a substrate uptake inhibitor and a specific amino acid residue side chain of a plasma membrane monoamine transporter protein have been unequivocally established, but findings for some of the above-discussed mutations make a compelling case for the proposed drug-protein association. The mutagenesis findings discussed here may serve to narrow the focus to specific TM domains in the search for inhibitor binding sites. TMs 1, 2, 3, 8, and 12 appear to be especially important contributors to inhibitor binding sites, but it should be noted that some of the remaining TM domains may not have been studied as rigorously in this respect. Monoamine transporter chimeras have implicated TMs 5 through 8 in inhibitor selectivity,[69,70,106] suggesting a closer inspection of TMs 5 through 7 is in order. Covalent cross-linking of radiolabeled photoaffinity

ligands to the DAT followed by proteolytic and immunological peptide mapping revealed that GBR-12,909 and benztropine photoaffinity analogs labeled a fragment containing TMs 1 and 2, while the cocaine analog RTI-82 labeled a fragment containing TMs 4 through 7.[107-109] By expanding the arsenal of photoaffinity probes used and employing mass spectrometry of high-performance liquid chromatography (HPLC)-purified proteolysis fragments, this methodology should eventually identify the specific cross-linked amino acid side chains of the transporter protein.[110-112] Assuming that the alkyl chain tether of the photoaffinity probe is sufficiently short to ensure that the cross-linked transporter side chain lines the functional binding site of the uptake inhibitor, this approach may be the most promising in elucidating sites of action of cocaine, amphetamine, and antidepressants. Continuing refinement of structure-function methods and molecular modeling templates for the monoamine transporter proteins will likely yield superior medications in combating psychostimulant abuse and monoamine neurotransmitter-related psychiatric disorders.

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